XPS characterization of surface films formed on surface-modified implant materials after cell culture

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Nitrogen ion-implanted Ti–6Al–4V, Ti–5Al–2.5Fe and 316 L stainless steel and nitrogen or carbon sputter-coated samples were inoculated with rat bone marrow. The interface between the cell layer and the substrata was studied by X-ray photo-electron spectrometry and observed by scanning electron microscopy (SEM). Ca and P were detected on all materials after *in vitro* cell culture. Titanium appears to be present mainly in the form of TiO_2.

1. Introduction

The surface characteristics of biomaterials play an important role in tissue response. The objective of modifying the surface characteristics of a biomaterial by means of a surface treatment, such as ion implantation and sputter coating, is to improve the corrosion and wear resistance as well as its biocompatibility. Ion implantation has been successfully used to promote wear resistance [1], corrosion resistance [2] and biocompatibility [3].

The study of the interface between living tissue and biomaterial is of crucial importance in understanding the mechanisms of cell bonding. Parameters such as surface roughness, oxide composition and thickness and the existence of contaminants on the surface are known to affect the biological response both *in vivo* and *in vitro* [4–7], thereby determining the success of cell attachment.

An osteoblast-cell culture was used to mimic the bone/biomaterial interface. This interface has been investigated *in vitro* by Davis *et al.* [8] and de Bruijn *et al.* [9] on titanium and calcium phosphates. They reported that the interface produced consists of a layer of Ca- and P-rich globular deposits adjacent to the implant surface over which collagen fibres are deposited. A similar interface was also observed to have been created *in vivo* [10].

The work here described is part of a study in which titanium alloys and stainless steel were surface modified by ion-implantation and sputter-coating techniques in order to improve their performance as implant materials. The surface-modified materials were previously characterized according to their corrosion behaviour, and the surfaces that produced better *in vitro* corrosion resistance were tested for biocompatibility. The objective of this study is the surface characterization of nitrogen ion-implanted and sputter-coated materials after rat bone marrow cell culture. Scanning electron microscopy (SEM) was used to observe the surfaces of the materials and the tissue immediately adjacent. The chemical composition of the substrata was determined by X-ray photoelectron spectrometry (XPS).

2. Materials and methods

2.1. Materials

Ti–6Al–4V and Ti–5Al–2.5Fe samples (Deutsch Titan) were cut from rods of 15 and 30 mm in diameter, respectively, and were ion implanted with two different fluences of nitrogen ions, namely: 10^{15} and 10^{16} ions/cm^2 with a beam energy of 40 keV. 316 L stainless steel (Aubert & Duval) samples were obtained from a rod of 20 mm in diameter and nitrogen ion-implanted with 10^{16} and 10^{17} ions/cm^2 with the same energy.

Samples of 316 L stainless steel (Aubert & Duval) were sliced from rod of 20 mm in diameter and then nitrogen- or carbon-sputter coated.

All samples were ultrasonically cleaned in 90% ethanol for 20 min, followed by a 20 min double rinse with distilled water before steam sterilization [11].

2.2. Cell culture

The samples were steam sterilized before cell culture for 20 min. A primary droplet rat bone marrow culture (RBMC) was performed according to the method described by Maniatopoulos *et al.* [12] which induces an osteoblast-enriched cell population.

Young adult male Wistar rats, of about 100–120 g were sacrificed. The epiphyses were removed and the bone marrow was flushed out from each dyaphise with α-minimum essential medium (Gibco), 15% foetal calf serum (Gibco) and 10% antibiotics (amphotericin B (Sigma), penicillin G (Sigma) and gentamycin

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(Gibco)). The cells were then grown on this medium to which 1% 1 M Na-β-glycerophosphate (Sigma), 1% 5 mg/ml ascorbic acid (Sigma) and 1% 10^{-8} M dexamethasone (Sigma) were added. A droplet culture of the bone marrow cell suspension was placed on every sample. The cell culture was performed either in 6- or 24-well tissue culture plates (Costar) depending on the sample dimensions and kept in a humidified atmosphere of 95% air–5% CO_2 at 37 °C. The cultures were observed with a light microscope every 48 h. The cultures were maintained for 19 days and then the samples were prepared for XPS analysis.

Samples were also prepared for scanning electron microscopy (SEM). Briefly, the specimens were fixed in 1.5% glutaraldehyde followed by dehydration in a graded series of ethanol and critical point drying (Balzers CPD 030) from carbon dioxide. The specimens were gold-sputter coated and observed by SEM with an accelerating voltage of 15 kV.

2.3. X-ray photo-electron spectrometry

After the 19 days of cell culture, the samples were thoroughly washed in Physiological Body Solution (PBS) and the cell layer was scraped with a rubber policeman. The samples were then washed twice with PBS and rinsed in distilled water. A VG Scientific ESCALAB, using MgK_α as source, was used to obtain the spectra. Survey XPS spectra from 0 to 1100 eV were acquired after cell culture on all surfaces. Survey spectra were also acquired on surface-modified materials which were not subjected to cell culture in order to provide comparison data. Following spectra acquisition, peak identification and quantification were achieved using VG Scientific ESCALB package software. High-resolution spectra for the Ca 2p, P 2p, O 1s, Ti 2p and C 1s were also obtained and computer curve-fitted employing a Gaussian model, using the same package software, to obtain the best binding energy (BE) values. To take into account some shift caused by charging of the sample surface, all spectra were adjusted taking the C 1s peak at 285.0 eV as reference for the adventitious carbon contamination.

3. Results and discussion

Wide scans were performed on nitrogen ion-implanted and sputter-coated samples before and after RBMC. C and O, as well as the alloy elements, were detected in all samples before cell culture. On the titanium alloys V was not observed as this element does not contribute to the formation of the surface film [13, 14]. Si, probably having its origin in the sample preparation process, was a common contaminant on all ion-implanted samples before cell culture. F was detected on the ion-implanted titanium alloys before RBMC. After cell culture, Al, Si and F signals were no longer detected. An exception was noticed for the Ti–6Al–4V ion-implanted samples with 10^{16} ions/cm^2 where F was detected after RBMC but the peak height was reduced by around 75%, which implies a reduction in its concentration. Ca was detected as a contaminant on ion-implanted Ti–5Al– 2.5Fe samples. After cell culture, Ca and P were easily detected on all materials investigated. N, also present on all samples before RBMC, showed an enhanced signal after cell culture, indicating that part of the N is of biological origin. A similar result may be deduced from the width analysis of the C 1s peak. The peak broadening after cell culture suggests that there are contributions from more chemical states than is the case for the usual C contamination. Ti concentration on the titanium alloys and Fe concentration on the stainless steels were reduced after cell culture. Ni and Cr, detected on the ion-implanted 316 L stainless steel before cell culture, were not seen after RMBC. Ni and Cr peaks were easily observed on all sputter-coated materials before and after cell culture.

Other groups [13, 15–17] have studied the surface composition of Ti cp, Ti–6Al–4V by XPS. Ti, O and C were always detected. Ca and traces of S, Cl, P, Si, Na, Cu, Zn, Sn and Pb were also found on Ti cp. samples before sterilization. Surface analysis of *in vivo* retrieved titanium [18, 19] and stainless steel samples [18] showed that Ca and P were always present. Similar results were obtained in this work by studying the surfaces after cell culture. Ca and P may have their origin from the cell-culture medium, as it is known that these elements are present on the surface after immersion of Ti–6Al–4V, Ti–5Al–2.5Fe and 316 L stainless steel in SBF or HBSS [13–15, 20]. Another probable origin of the Ca and P is related to the extra-cellular matrix produced by the osteoblast-like cells. SEM observations of the interface substrata/cell layer [21] showed that Ca- and P-rich globular deposits are produced by these cells as as soon as they colonize the material. Even after mechanically removing the cell layer, some of these globular accretions were still attached to the surface. While preparing the surfaces for XPS spectra acquisition the cell layer was scraped with a rubber policeman but the adhesion of the cell layer to the material surface, especially on ion-implanted titanium alloys, was very strong, leaving some cell-layer leftovers attached to the surfaces. Care was taken to choose the cleanest areas of the samples for spectra acquisition.

The reduction in concentration of most elements and the disappearance of the contaminants after RBMC may be a consequence of surface oxide thickening due to both cell contact and interaction with the cell-feeding media. By comparing the morphologies of the different cell layers produced on the surface-modified materials, one notices that the cell response to the sputter-coated materials was worse compared to that of ion-implanted ones. The cell layer grew heterogeneously on these surfaces and on nitrogen sputter-coated samples an extra-cellular matrix was not produced even after 19 days of culture. The detection of alloy elements such as Cr and Ni after cell culture may explain these results since the toxicity of these elements is well known [22, 23].

High-resolution spectra were acquired, on nitrogen ion-implanted Ti–6Al–4V, for C 1s, O 1s, Ti 2p, P 2p and Ca 2p. This provided an indication of the chemical composition of the calcium phosphate and the titanium oxide formed on the surfaces. The

high-resolution spectra were very similar for both fluences. The binding energies obtained differed by max. $\pm$ 0.2 eV. Figs 1 to 5 show the high-resolution spectra C 1s, O 1s, Ti 2p, P 2p and Ca 2p.

C 1s spectra. The spectra were dominated by a shoulder at high energies and it was necessary to employ two peaks to achieve the best fit. The main peak had a binding energy (BE) of 288.2 $\pm$ 0.1 eV, which indicates that the carbon is bonded to O or hydroxyl groups [15]. The full width half maximum (FWHM), of the order of 2.7 eV for the lower binding-energy peak and 2.5 eV for the higher binding-energy peak, suggests that there are overlapping peaks. Therefore carbon may be present as a mixture of compounds, probably as sodium or calcium carbonates since C 1s shows standard binding energies for these compounds of 289.4 eV and 289.8 eV, respectively [24]. Nevertheless, the main source of contamination is probably due to organic molecules having an origin either in the cell culture or simply in the exposure to air.

O 1s spectra. The main peak has a BE of 534.6 $\pm$ 0.0 eV and a FWHM of 2.73 $\pm$ 0.0 eV which indicates, as for the C 1s peak, the overlapping of more than one peak. A shoulder at lower BE (531.2 eV) was also detected. Armstrong *et al.* [25] determined the standard O 1s for TiO_2 = 533.3 eV which is similar to the BE of the main peak. Binding energy differences between two major transitions can also indicate the chemical composition of a metal oxide. By calculating the BE difference between the O 1s and the Ti 2p 3/2, ΔBE = O (1s) – Ti (2p 3/2) = 75.1 eV which, according to the data obtained by the same authors, corresponds to TiO. On the other hand, high-resolution spectra obtained by other groups [14–16, 26] suggest that the BE for the oxide appears in the low-energy range ($\approx$ 531 eV) followed by other components at higher BE, namely hydroxide/hydroxyl groups, phosphate or chemisorbed water. Taking this into account, it is possible that titanium oxide contributes mainly to the O 1s peak at BE 531.2 eV, and that the main peak at 534.6 eV can be mostly attributed to the presence of phosphates and adsorbed water. Recalculating ΔBE one obtains the value of 71.7 eV which is closer to the values obtained by Armstrong *et al.* [25] for TiO_2. The analysis of the Ti 2p spectra also suggests that the titanium is mostly present in the form of TiO_2.

Ti 2p spectra. The Ti 2p peak was a doublet peak, Ti 2p3 at 459.5 $\pm$ 0.1 eV and Ti 2p1 at 465.0 $\pm$ 0.2 eV. This indicates that the titanium is present mainly in the oxide state [22] and by taking into account the BE of the peaks [16, 24] and the separation between them [24] it is probable that the oxide present is TiO_2 which is in accordance with results from the O 1s spectra. No contributions were detected between the main peak and 454.0 eV which is BE value for Ti metal. This suggests first, that the surface film is too thick to allow photo-electrons from the bulk metal to reach the surface, and second, that there are no sub-oxides, such as TiO and Ti_2O_3, present [15, 23].

P 2p spectra. This was a single peak with a BE of 136.0 $\pm$ 0.1 eV and a FWHM of 2.39 eV indicating a probable overlapping peak. According to the literature [13, 14, 22, 24, 27, 28] there is no clear indication as to the nature of the chemical compound in which this element is present. A probable mixed Ca and P compound may be responsible for its high BE.

Ca 2p spectra. This was a doublet peak with Ca 2p 1/2 = 353.8 $\pm$ 0.1 eV and Ca 2p3/2 = 350.2 $\pm$ 0.1 eV. The FWHM of Ca 2p 3/2 is around 2.1 eV which is relatively large for Ca which has a value of 1.68 eV [22]. This suggests that the peak overlaps with other contributions. As for the P 2p, Ca 2p 3/2 has a BE which enables the identification of the Ca compound. Comparison of the BEs of P 2p and Ca 2p 3/2 suggests that a Ca- and P-compound may have been formed, probably a calcium-hydrogen phosphate.

It is possible that the features obtained in the XPS spectra are mainly a consequence of the Ca- and

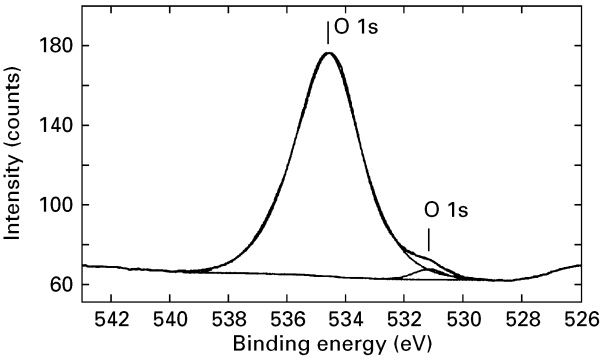

Figure 2 XPS O 1s high-resolution spectrum from an N^+-ion implanted Ti–6Al–4V with 10^{15} ions/cm^2 sample.

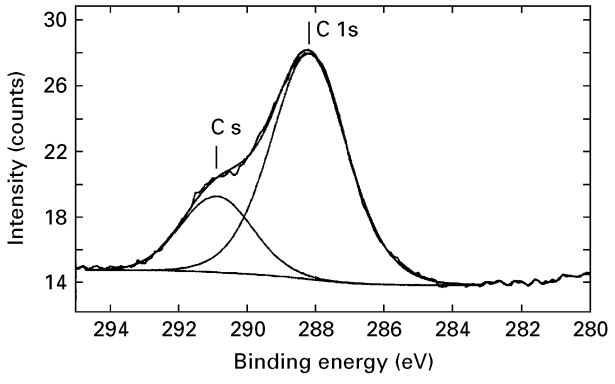

Figure 1 XPS C 1s high-resolution spectrum from an N^+-ion implanted Ti–6Al–4V with 10^{15} ions/cm^2 sample.

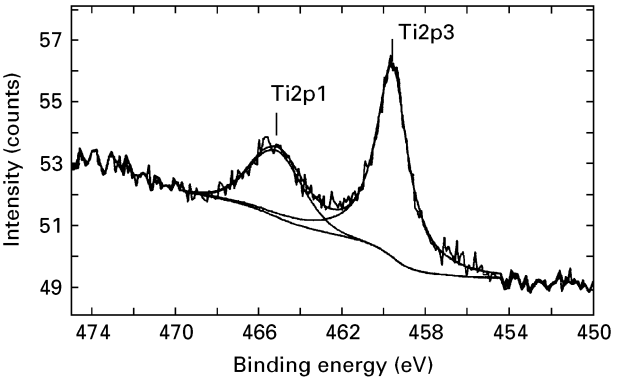

Figure 3 XPS Ti 2p high-resolution spectrum from an N^+-ion implanted Ti–6Al–4V with 10^{15} ions/cm^2 sample.

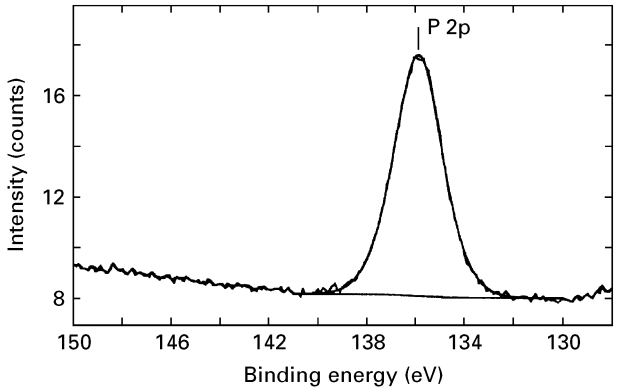

Figure 4 XPS P 2p high-resolution spectrum from an N^+-ion implanted Ti–6Al–4V with 10^{15} ions/cm^2 sample.

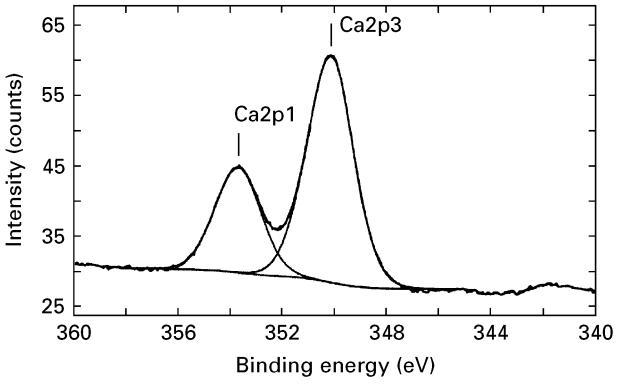

Figure 5 XPS Ca 2p high-resolution spectrum from an N^+-ion implanted Ti–6Al–4V with 10^{15} ions/cm^2 sample.

P-rich globular deposits formed by the osteoblast-like cells. As mentioned before, it is also possible that a calcium phosphate may have been formed as a result of the immersion of the samples in the cell-feeding medium which is composed partly of compounds of Ca and P. A third possibility is the joint effect of both osteoblast-like cells and medium.

4. Conclusions

(1) *In vitro* cell culture seems to be a fairly reliable method to test biomaterials *in vitro* because the interface formed is both morphologically and chemically similar to those observed *in vivo*.

(2) The bad cell culture results exhibited by the sputter-coated 316 L stainless steel seem to result from the existence of Cr and Ni ions on the metal surface.

(3) SEM observations of the interface between the cell layer and the substrata together with the XPS results suggest that the surface of the nitrogen ion-implanted Ti–6Al–4V is mainly composed of TiO_2 and a biologically produced calcium-phosphate compound.

Acknowledgements

This work was carried out under Project BRITE/EURAM nr. 91-0477. The authors thank Laboratoire PHASE-CNRS, Strasbourg and Laboratoire de Sciences et Génie des Surfaces, Nancy for providing the ion-implanted and sputter-coated samples, respectively. E. Leitão was supported by a grant from the Ciencia/Praxis programmes.

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Received 2 July
and accepted 7 August 1996